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A nonerythropoietic peptide derivative of erythropoietin decreases susceptibility to diet-induced insulin resistance in mice.

M. Collino^{1*}, E. Benetti¹, M. Rogazzo¹, F. Chiazza¹, R. Mastrocola², D. Nigro², J. C. Cutrin^{3,4}, M. Aragno², R. Fantozzi¹, M. A. Minetto⁵, C. Thiernemann⁶

¹ *Department of Drug Science and Technology, University of Turin, Turin, Italy*

² *Department of Clinical and Biological Sciences, University of Turin, Turin, Italy*

³ *Department of Biotechnology and Sciences for the Health, University of Turin, Italy;*

⁴ *ININCA-CONICET, Argentina*

⁵ *Division of Endocrinology, Diabetology and Metabolism, Department of Medical Sciences, University of Turin, Turin, Italy*

⁶ *Queen Mary University of London, Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, UK*

***Corresponding author:**

Massimo Collino, Department of Drug Science and Technology, University of Turin,
via P. Giuria 9, 10125 Torino, Italy.

Tel: +39 011 6707955 Fax: +39 011 2367955

E-mail address: massimo.collino@unito.it

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Summary

Background and purpose: the hematopoietic activity of erythropoietin (EPO) is mediated by the classic EPO receptor (EpoR) homodimer, whereas the tissue-protective effects by a hetero-complex between EpoR and the β -common receptor (β cR). Here, we investigated the effects of a novel, selective ligand of the hetero-complex EpoR/ β cR (pyroglutamate helix B surface peptide [pHBSP]) in mice chronically exposed to a diet enriched in sugars and saturated fats.

Experimental approach: male C57BL/6J mice maintained on high-fat high-sucrose diet (HFHS) for 22 weeks. pHBSP (30 μ g/kg s.c.) was administered for the last 11 weeks.

Key results: mice fed the HFHS diet exhibited insulin resistance, hyperlipidemia, hepatic lipid accumulation and kidney dysfunction. In the gastrocnemius muscle, HFHS impaired the insulin-signaling pathway and reduced GLUT-4 membrane translocation and glycogen content. Administration of pHBSP ameliorated renal function, reduced hepatic lipid deposition and normalized serum glucose and lipid profiles. These effects were associated with a significant improvement in insulin sensitivity and glucose uptake in the skeletal muscle. Diet-induced overproduction of the myokines interleukin-6 and fibroblast-growth-factor-21 were attenuated by pHBSP and, most importantly, pHBSP markedly enhanced muscle mitochondrial biogenesis.

Conclusions and implications: this is the first report that chronic treatment with an EPO-derivative devoid of any hematopoietic effects triggers an amelioration of the HFHS-induced metabolic abnormalities by affecting multiple levels of the insulin signaling and inflammatory cascades within the mouse skeletal muscle while enhancing mitochondrial biogenesis.

Keywords: *erythropoietin, pyroglutamate helix B surface peptide, β -common receptor, high-fat high-sucrose diet, insulin resistance, insulin signaling pathway, myokines, inflammation*

Abbreviations: *β cR: β -common receptor; ACR: albumin-to-creatinine ratio; BUN: blood urine nitrogen; COI: cytochrome c oxidase 1; EPO: erythropoietin; EpoR: EPO receptor; GLUT-4:*

glucose transporter type 4; HFHS: high-fat high sugar; mtTFA: mitochondrial transcription factor A; NRF1: nuclear respiratory factor 1; OGTT: oral glucose tolerance test; PGC1- α : peroxisome proliferator-activated receptor γ co-activator 1- α ; pHBSP: pyroglutamate helix B surface peptide; PVDF: polyvinylidenedifluoride; TG: triglyceride

INTRODUCTION

Erythropoietin (EPO), a 31 kDa glycoprotein that stimulates proliferation, differentiation and survival of erythroid progenitor cells by activation of the EPO receptor (EpoR), has been widely used for the treatment of chronic anemia (Drueke *et al.*, 2006). A large number of experimental studies demonstrate a tissue-protective effect of EPO in the brain, the kidney, the heart, the vasculature and the gastrointestinal tract, to name but a few (Patel *et al.*, 2011). Interestingly, EPO deficiency has been documented in patients with diabetes (Craig *et al.*, 2005; McGill *et al.*, 2006; Thomas, 2006), and EPO therapy has been reported to decrease insulin resistance in hemodialysis patients by improving glucose metabolism and reducing chronic inflammation (Khedr *et al.*, 2009; Mak, 1998; Rasic-Milutinovic *et al.*, 2008; Spaia *et al.*, 2000). Collectively, these data raised the possibility that EPO and the signaling cascades activated by the pleiotropic hormone may significantly affect insulin sensitivity and, thus, may be relevant for management of diabetes. Potentially serious adverse effects of EPO including an increase in hematocrit, blood pressure and thrombosis may limit any potential therapeutic translation of these beneficial effects of EPO in diabetic patients. Although the EPO-mediated signaling pathways are well characterized for erythroid cell types, they are less well defined for non-erythroid tissues and, therefore, there is limited information about the mechanisms underlying the EPO-induced improvements in insulin resistance. The extra-hematopoietic effects of EPO have been suggested to be mediated, at least in part, by a putative “tissue-protective receptor”, that has been recently proposed to be a hetero-complex between the EpoR and the β -common receptor (β cR, known also as CD131) (Brines *et al.*, 2004). β cR is a common subunit of other heteroreceptors, including those of interleukin (IL)-3, IL-5 and granulocyte-macrophage colony stimulating factor (Murphy *et al.*, 2006). Very recently, the use of β cR knockout mice has led to the demonstration that the activation of β cR by EPO is essential for the EPO-induced reduction of both kidney and cardiac dysfunction associated with sepsis, as well as in producing long-term relief of neuropathic pain (Coldewey *et al.*, 2013; Khan *et al.*, 2013; Swartjes *et al.*, 2011). Moreover, the development of EPO-derivatives, which only activate the

EpoR- β cR complex and do not stimulate erythropoiesis, has led to a better characterization of the role of this pharmacological target in mediating EPO effects (Leist *et al.*, 2004). The pyroglutamate helix B surface peptide (pHBSP), also known as ARA290, is the newest generation EPO derivative, which mimics the external, aqueous face of helix B of EPO (including amino acids that are exposed at the helix B surface as well as three residues from the loop between helices B and C), sufficiently to exert the tissue-protective activities seen with the EPO protein. Thus, pHBSP is a small synthetic peptide consisting of eleven amino acids, which binds to the EpoR- β cR complex, but not the classical erythropoietic EpoR homodimer in vitro (Brines *et al.*, 2008a). Several preclinical studies have revealed that pHBSP exerts pronounced tissue-protective properties without stimulating hematopoiesis (Brines *et al.*, 2008b; McVicar *et al.*, 2011; Pulman *et al.*, 2013; Seeger *et al.*, 2011; Swartjes *et al.*, 2011; van Rijt *et al.*, 2013). However, none of the previous studies investigated the role of the EpoR- β cR complex activation in the management of metabolic abnormalities or any other chronic disease where administration of the drug for several weeks is warranted. Hence, the present study was undertaken to determine the effects of the non-erythropoietic EPO derivative pHBSP in mice chronically exposed to a diet enriched in sugars and saturated fats, which are known to promote obesity and insulin resistance. To provide a characterization of the extra-hematopoietic effects of EPO, we investigated the potential effects of the activation of the EpoR- β cR complex by pHBSP on signaling pathways involved in the pathogenesis of insulin resistance, focusing on the skeletal muscle, which accounts for ~70–80% of the insulin-stimulated glucose uptake and, thus, exerts a key role in regulating whole body glucose homeostasis.

METHODS

Animal model

Male C57BL/6J mice, aged 4 weeks, were purchased from Harlan Laboratories (Udine, Italy). The animals were maintained on a pellet diet (Piccioni n.48, Gessate Milanese, Italy) for 1 week, then randomly divided into two groups: normal diet (control, $n = 18$) and high-fat high sugar diet (HFHS, $n = 24$) for 22 weeks. The HFHS diet contained 40% fat (2% from soybean, 38% from butter), 15% protein, 45% carbohydrate (15% from corn starch and 30% from sucrose). The animals were housed in a temperature-controlled environment with a 12 h light/dark cycle. Food and water consumption and body weight were measured weekly. The experimental protocol was approved by the Turin University Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Treatments

After 11 weeks on diet, 6 mice on control diet and 12 mice on HFHS diet were treated with pHBSP (30 $\mu\text{g/kg}$, in 200 μl PBS) for further 11 weeks (control+pHBSP and HFHS+pHBSP, respectively). The EPO derivative pHBSP was supplied by Araim Pharmaceuticals (Ossining, NY, USA). Mice were dosed by s.c. injection at two-day interval. Vehicle treatment consisted of 200 μl PBS at pH 7.4. The pHBSP dose used in this study is based on previous studies on tissue protective effects of chronic pHBSP administration in mice (Brines *et al.*, 2008b; Schmidt *et al.*, 2011; Swartjes *et al.*, 2011). This dosage of pHBSP was previously found to induce no change in the hematocrit, hemoglobin concentration and platelet count over a 28-day period of twice daily administration (Brines *et al.*, 2008b). The interval of drug administration was based on published preclinical and

clinical data (Heij *et al.*, 2012; Swartjes *et al.*, 2011; van Velzen *et al.*, 2014), which clearly demonstrate that the repetitive (2-day intervals) i.p. administration of pHBSP exerts significant beneficial effects, despite its short half-life ($t_{1/2 \text{ ELIM}} \sim 2 \text{ min}$).

Oral glucose tolerance test (OGTT).

OGTT was done on mice fasted for 6 h at the end of the experiment (week 22). Glucose (2 g/kg) was given orally by gavage. Serial blood glucose measures were taken at 0, 30, 60, 90, and 120 min from the saphenous vein using the Accu-Check glucometer (Roche Diagnostics, Germany).

Biochemical parameters.

Urine was collected for analysis by placing animals in metabolic cages for 18 h. The concentration of urine creatinine and albumin was assessed using commercial kits (Arbor Assays, USA and Bethyl Laboratories Inc., USA, respectively). Albumin excretions were related to urine creatinine concentrations (albumin-to-creatinine ratio, ACR) in order to take into account differences in urinary flows. Blood urea nitrogen (BUN) levels as well as plasma concentrations of triglycerides, total cholesterol, high-density-lipoprotein (HDL), and low density-lipoprotein (LDL) were determined using colorimetric assays from Sigma Chemical (USA) and Hospitex Diagnostics (Italy), respectively. Commercially available ELISA kits were used to measure plasma insulin, leptin, adiponectin, IL-6 and FGF-21 levels.

Kidney histopathological examination

Coronal sections of both kidneys and fragments from the left lateral and medial lobes were fixed in 4% buffered formaldehyde solution overnight at 4° C. Dewaxed 5 µm sections were stained with

hematoxylin-eosin and examined under an Olympus Bx4I microscope (40x magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

Liver Oil Red staining

Neutral lipids were assessed on sections of frozen liver embedded in OCT (10 µm in thickness) by Oil Red O staining using an Olympus Bx4I microscope (40x magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

Protein extraction and western blot

Gastrocnemius and liver were homogenized and fractioned as previously described (Benetti et al., 2013). Briefly, frozen tissues were homogenized in RIPA buffer containing protease inhibitors. The protein content was measured by a protein assay kit using bicinchoninic acid and albumin as standard (Pierce Biotechnology Inc., USA). Protein-matched samples were electrophoresed, and then transferred to a polyvinylidenedifluoride (PVDF) membrane, which was then incubated with primary antibodies (GLUT-4, COI, PGC-1 α , mtTFA, NRF-1, and the total and phosphorylated forms of IRS-1, Akt, GSK-3 β and AS160; Santa Cruz Biotechnology Inc.). After incubation with primary antibodies, membranes were incubated with secondary antibodies linked to horseradish peroxidase. Protein bands were visualized using enhanced chemiluminescence. For control of protein loading, the blots were stripped and reacted with tubulin monoclonal antibody. Bands were quantified by an optical densitometry using Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA).

Liver triglyceride (TG) level

Lipids were extracted from liver tissue as previously described (Collino *et al.*, 2013) and TG contents were determined enzymatically by using a TG quantification kit from Abnova Corporation (Germany).

Skeletal muscle glycogen content.

The glycogen concentration of the gastrocnemius was determined using a glycogen assay kit, following the protocol provided by the manufacturer (Glycogen Assay Kit, Abnova Corporation, Taiwan).

EpoR/ β cR co-immunoprecipitation.

Co-immunoprecipitation was performed in order to examine whether there is a physical interaction between EpoR and β cR. Protein extracts were immunoprecipitated from gastrocnemius lysates using EpoR antibody for 3 hours, followed by incubation with protein A-agarose beads overnight at 4°C. The mixture was centrifuged for 5 minutes and supernatants were removed. Bead-antibody-protein complexes were washed in RIPA buffer and proteins were eluted from protein A-agarose beads by boiling in loading buffer. After centrifuge antigen-antibody complexes were subjected to SDS-PAGE using 8% gel, followed by transfer to PVDF membranes. The membranes were incubated with the primary antibodies anti-EpoR or anti- β cR and the appropriate secondary antibodies.

Immunohistochemical staining

Representative 10- μ m cryostat sections of gastrocnemius were fixed with 100% acetone for 10 min and then incubated with rabbit anti-glucose transporter type 4 (GLUT-4) antibody overnight at 4°C.

After washing, sections were incubated with anti-rabbit IgG-HRP for 1h at room temperature. Color development was achieved by incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 minutes at room temperature and the specific staining visualized with Olympus-Bx4I microscope connected by a photographic attachment (Carl Zeiss, Oberkochen, Germany).

Statistical analysis.

We analyzed data using the Prism software package (GraphPad Software). Comparisons among groups were performed using one-way analysis of variance with Bonferroni's multiple comparison post hoc test. All values are presented as mean \pm S.E.M. for n observations. Differences among groups were considered statistically significant at p values below 0.05.

RESULTS

Effects of diet exposure and pHBSP treatment on metabolic parameters.

After 22 weeks of feeding, mice of the HFHS group had greater body and adipose tissue (epididymal fat) weights than control diet-fed animals, which were significantly reduced by pHBSP treatment (Table 1). Moreover, an increase in the caloric intake in the mice fed with the experimental diet was recorded and this increase was slightly reduced by drug treatment without reaching statistical significance. As reported in Table 2, HFHS increased serum levels of triglycerides, total cholesterol and LDL and reduced HDL level, in comparison with control mice. Most notably, the changes in lipid contents were normalized by pHBSP treatment. When compared to control, chronic exposure to HFHS resulted in more than two-fold increase in insulin levels, which was associated with almost 30% increase in fasting serum glucose concentrations (Figure 1A and 1B). Consistently, the OGTT revealed that the HFHS mice were glucose intolerant (Figure 1C). In contrast, pHBSP administration exerted significant insulin- and glucose-lowering ability. Serum concentrations of adiponectin and leptin, both of which play important roles in lipid and glucose homeostasis, showed different patterns: while the level of adiponectin in HFHS mice was lower than in control mice, that of leptin increased versus controls (Table 2). When pHBSP was administered to HFHS mice, there was a significant increase in the adiponectin levels, associated with a significant decrease in leptin levels, while pHBSP administration to mice fed with the control did not modify any of the above described metabolic parameters.

Chronic pHBSP treatment reduces diabetic nephropathy in HFHS mice.

Representative pictures from each experimental group were taken from the transition between the deeper cortex and the outer stripe of the outer medulla zones (Figure 2A). Kidneys from mice fed with a standard laboratory chow showed a normal appearance and treatment of control mice with pHBSP did not produce any significant morphological alterations. In contrast, the HFHS diet

produced an intense vacuolar degeneration of the S1 and S2 portions of the proximal convoluted tubules, which may be the consequence of specific features of these segments, including prominent endocytic system and large secondary lysosomes (Maunsbach, 1966). In contrast, the normal appearance of the S3 segments (Supplemental Figure 1) could be the result of a lesser development of the endocytic vacuolar system of the “pars recta”. Interestingly, the HFHS-induced alterations were significantly attenuated by pHBSP treatment. The HFHS-induced renal pathology correlated with decline in kidney function. The ACR ratio, a reliable marker of glomerular damage and progressive renal dysfunction associated with diabetes and obesity (Praga *et al.*, 2010), was remarkably elevated in HFHS-fed mice and significantly reduced after pHBSP administration for 11 weeks (Figure 2B). Similarly, BUN levels were markedly increased in the HFHS group compared with the control group and significantly reduced by pHBSP treatment (Figure 2C)

Chronic pHBSP treatment reduces liver steatosis.

Livers from control mice showed a normal appearance, and chronic treatment with pHBSP had no effect on liver histology. In contrast, livers from mice that had received the HFHS diet showed moderate to severe mixed vacuolar steatosis (Figure 3A and Supplemental Figure 2). The observed steatosis was more prominent in the periportal zone, where hepatocytes exhibited some degree of hydropic degeneration. Chronic administration of pHBSP attenuated the neutral fat accumulation (measured after Oil Red O staining; Figure 3A). This beneficial effect of pHBSP on lipid accumulation was confirmed by measurement of TG content (Figure 3B). Liver homogenates from HFHS mice showed a 3-fold increase in TG levels when compared to control animals. Most notably, the TG levels of HFHS animals that had been treated with pHBSP were similar than those seen in control animals.

Insulin signal transduction was enhanced in gastrocnemius muscle of HFHS mice exposed to pHBSP treatment.

In the skeletal muscle, the expression levels of total IRS-1, Akt and GSK-3 β proteins were not affected by either dietary manipulation or drug treatment (Figure 4). In contrast, 22 weeks of HFHS diet caused a marked increase in Ser³⁰⁷ phosphorylation of IRS-1 in parallel with reduced Ser⁴⁷³ phosphorylation of Akt and Ser⁹ phosphorylation of GSK-3 β , a downstream target of Akt, thus indicating impaired insulin signaling pathway. Chronic pHBSP administration significantly counterbalanced all the effects of HFHS diet on IRS-1, Akt and GSK-3 β phosphorylation.

GLUT-4 and glycogen levels in the mouse skeletal muscle.

As shown in Figure 5B, GLUT-4 expression was slightly (although not significantly) reduced in gastrocnemius of HFHS mice compared to control animals, and increased following pHBSP administration. Interestingly, pHBSP administration not only increased carrier expression levels, but also induced a significant membrane translocation, as shown by a different pattern of GLUT-4 immunostaining between muscle sections of mice exposed to dietary manipulation in the absence or presence of pHBSP (Figure 5A). GLUT-4 translocation to the plasma membrane was confirmed by Western blot analysis of the Akt substrate 160 (AS160), a protein that regulates insulin-stimulated GLUT-4 trafficking in skeletal muscle (Kramer *et al.*, 2006). AS160 phosphorylation is required for GLUT-4 translocation to the cell surface. Here we showed that the total protein abundance of AS160 in the mouse gastrocnemius was not affected by either dietary manipulation or drug treatment. However, Thr⁶⁴² phosphorylation of AS160 was significantly reduced by HFHS diet and this effect was prevented by pHBSP administration (Figure 5C). The HFHS-induced modulation of GLUT-4 membrane translocation was accompanied by a robust decrease in muscle glycogen content, which was blunted by pHBSP treatment (Figure 5D).

Administration of pHBSP prevented local and systemic myokine overproduction evoked by chronic consumption of HFHS.

HFHS significantly elevated skeletal muscle production of two well-known myokines, IL-6 and FGF-21, while pHBSP treatment resulted in a massive decrease in IL-6 and a slight reduction in FGF-21 levels (Figure 6A and 6B, respectively). These changes in the muscle myokine production were paralleled by the changes in myokine circulating levels. In fact, circulating concentrations of IL-6 and FGF-21 increased after HFHS feeding and these increases were almost completely abolished in mice treated with pHBSP. In contrast, their levels were not altered by pHBSP treatment in mice fed with the control diet (Figure 6C and 6D).

Effect of chronic pHBSP treatment on markers of muscle mitochondrial production/biogenesis.

To get further insight toward the effects of pHBSP, selective markers of muscle mitochondrial production/biogenesis were evaluated. Protein expression of the mitochondrial component of the electron transfer cytochrome c oxidase 1 (COI), used as a marker of mitochondrial content, and peroxisome proliferator-activated receptor γ co-activator 1- α (PGC1- α), a transcriptional coactivator mediating mitochondrial biogenesis, increased in the gastrocnemius muscle of animals fed with the HFHS diet (Figure 7A and 7B, respectively). Interestingly, a further increase in PGC1- α expression was observed in the muscle of HFHS mice treated with pHBSP. Moreover, expression of the mitochondrial transcription factor A (mtTFA) and nuclear respiratory factor 1 (NRF1), which act in concert to increase mitochondrial oxidative phosphorylation and mitochondrial biogenesis, were not affected by HFHS diet, whereas they were markedly up-regulated by pHBSP (Figure 7C and 7D, respectively).

Evidence of an heteromeric complex consisting of EpoR and β cR in the mouse skeletal muscle.

Immunoprecipitation assay showed that EpoR and β cR were physically associated in the gastrocnemius muscle of control animals and this interaction was not altered in response to dietary modification and/or pHBSP treatment (Figure 8).

DISCUSSION

The current study demonstrates for the first time that chronic administration of the EPO-derivative pHBSP protects against the metabolic abnormalities caused by exposure to a typical unhealthy diet containing high concentrations of both fat and sugar. Mice fed with a HFHS diet for 22 weeks developed a significant increase in body weight associated with dyslipidaemia, hyperinsulinaemia, and changes in insulin sensitivity and, most notably, treatment with pHBSP normalized serum glucose and lipid profiles and evoked a slight, but still significant, decrease in body weight, with reduced amounts of epididymal fat tissue. This decrease was not due to reduced food intake, as this parameter was measured and ruled out. In addition, we observed several pathophysiological alterations in kidney(s) and liver of mice that had been maintained on the HFHS diet, which were significantly attenuated by drug treatment. In HFHS-animals, pHBSP reduced the diet-induced diabetic nephropathy (confirmed by histology, rise in BUN levels and albuminuria). Similarly, pHBSP reduced the HFHS-induced liver steatosis (confirmed by histological analysis and determination of tissue TG levels). Overall, our data are in keeping with recent studies showing that EPO may ameliorate organ dysfunction evoked by metabolic derangements and regulate glucose metabolism and insulin sensitivity. Specifically, treatment of diabetic mice with EPO attenuated both albuminuria and podocyte loss (Loeffler *et al.*, 2013) and improved mitochondrial oxidative phosphorylation and lipid metabolism in the liver (Meng *et al.*, 2013). Interestingly, transgenic mice constitutively overexpressing human EPO are resistant to high fat diet-induced glucose intolerance and insulin resistance (Hojman *et al.*, 2009; Katz *et al.*, 2010). These data are supported by clinical evidence documenting beneficial effects of EPO on both glucose metabolism and insulin sensitivity in both diabetic and non-diabetic patients undergoing hemodialysis (Allegra *et al.*, 1996; Brown *et al.*, 2009; Khedr *et al.*, 2009; Tuzcu *et al.*, 2004). However, thus far any potential therapeutic applications of human recombinant EPO in patients with metabolic diseases are limited by rises in hematocrit and platelet count (hematopoietic effect), resulting in an increase in thrombotic risk. The EPO-mimetic pHBSP, which was evaluated in our study, binds selectively to the heteromeric

receptor composed of EpoR and β cR, which is responsible for the tissue-protective effects of EPO, but does not interact with the erythropoietic EpoR homodimer, activation of which triggers a rise in hematocrit (Brines *et al.*, 2008a). Therefore, our findings confirm the metabolic protective effects of EPO against diet-induced metabolic derangements and, most notably, demonstrate that these beneficial metabolic effects can also be obtained by selective activation of the EpoR- β cR complex, without stimulating hematopoiesis and, hence, potentially causing the severe side-effects associated with chronic EPO administration.

The skeletal muscle, which is a major site of postprandial glucose metabolism and one of the insulin-sensitive tissues most likely to manifest early signs of insulin resistance, is a main target of the protective effects of EPO. EPO is produced by primary myoblasts isolated from gastrocnemius muscle and it contributes to improve myoblast proliferation and survival as well as skeletal muscle repair and regeneration by activating the Akt signaling pathway (Jia *et al.*, 2012). EPO overexpression in skeletal muscle protects mice against diet-induced obesity and associated metabolic abnormalities (Hojman *et al.*, 2009). Davenport *et al.* also demonstrated that 14 weeks of EPO administration in patients with chronic kidney disease on hemodialysis increased muscle glycogen content (Davenport *et al.*, 1993), while in rats EPO induced a shift in muscle fiber composition from fast glycolytic fibers to slow oxidative fibers (Cayla *et al.*, 2008). Both EpoR and β cR are expressed in human skeletal muscle and are co-localized in the sarcolemma (Joshi *et al.*, 2013). Here we describe for the first time the physical interaction between EpoR and β cR in the mouse skeletal muscle by immunoprecipitation assay, showing that the pharmacological target of pH BSP, the EpoR/ β cR complex, is expressed in the skeletal muscle of both control and HFHS-fed mice. pH BSP is known to bind the EpoR/ β cR complex (Bohr *et al.*, 2013; Brines *et al.*, 2008a; Brines *et al.*, 2012) and no effect of pH BSP in mice lacking the β cR has been reported (Swartjes *et al.*, 2011; Swartjes *et al.*, 2013). However, further studies on the effects of pH BSP in β cR knock out

mice subjected to HFHS diet are warranted to confirm that the observed beneficial effects of this peptide are indeed due to activation of the EpoR/ β cR complex.

In our study, pHBSP attenuated the impairment in insulin signaling, caused by HFHS-diet in skeletal muscle, which may, at least in part, account for the changes in systemic insulin sensitivity. Specifically, pHBSP counteracted the HFHS-induced alterations in the phosphorylation of IRS-1 protein in the skeletal muscle, as well as the activities of the downstream key insulin signaling molecules, Akt and GSK-3 β , an Akt substrate. These effects were accompanied by a significant improvement in membrane translocation of GLUT-4, the most abundant glucose transporter isoform in skeletal muscle (Bouzakri *et al.*, 2006), and by a robust increase in muscle glycogen content. As the inhibition of GSK-3 β evokes an increase in the glycogen synthesis (Cross *et al.*, 1995), we speculate that the increased muscle glycogen storage evoked by pHBSP administration may be secondary to selective inactivation of GSK-3 β . Similarly, the pHBSP-induced membrane translocation of GLUT-4 could be secondary to the modulation of the insulin-signaling pathway by activation of EpoR/ β cR complex. Indeed, both Akt and IRS-1 may regulate the translocation, targeting and fusion of GLUT-4-containing vesicles in mice skeletal myocytes (Gonzalez *et al.*, 2006; Leto *et al.*, 2012; Zeng *et al.*, 2012). Akt also regulates AS160 phosphorylation, which is a crucial step in GLUT-4 translocation in the skeletal muscle (Kramer *et al.*, 2006; Sano *et al.*, 2003), and here we showed a significant increase in the phosphorylation of AS160 in the presence of pHBSP, which may result in GLUT-4 exocytosis and reduced GLUT-4 endocytosis. We propose that pHBSP stimulates glucose transport in skeletal muscle by increasing AS160 phosphorylation throughout Akt modulation, thus implying that the EpoR/ β cR complex is a common target of convergence to counteract both diet-induced impaired insulin signal transduction and glucose transport.

Compelling evidence has occurred over the past few years that the skeletal muscle is an endocrine organ that produces and releases a number of biologically active substances, such as IL-6, IL-8, IL-15, and FGF-21, collectively known as myokines (Pedersen *et al.*, 2008). These myokines

participate in cell-to-cell and organ-to-organ cross-talk and play important roles in the development of diseases associated with low-grade inflammation, including insulin resistance (Pedersen, 2011). Here we found that HFHS diet evoked up-regulation of IL-6 and FGF-21 in the skeletal muscle and that this effect was also associated with a strong increase in the plasma levels of IL-6 and FGF-21. Interestingly, administration of pHBSP led to dramatic reduction in the plasma levels of IL-6 and FGF-21. When measured in the gastrocnemius homogenates, however, the reduction in myokine levels by pHBSP was maximal for IL-6 and modest for FGF-21. This observation is intriguing, as the role of myokines in the etiology of insulin resistance has been matter of controversy. While chronic exposure to IL-6 produces insulin resistance in skeletal muscle both in vitro and in vivo (Nieto-Vazquez *et al.*, 2008), FGF-21 has emerged as an important regulator of glucose metabolism, increasing glucose uptake and abolishing insulin resistance in skeletal muscle cells (Ni *et al.*, 2012). In humans, serum FGF-21 levels are paradoxically increased in metabolic diseases (Chen *et al.*, 2008; Galman *et al.*, 2008; Han *et al.*, 2010). The mechanisms responsible for this paradoxical elevation of FGF-21 are not fully understood, although it might be partly explained by a compensatory overexpression of FGF-21 and/or FGF-21 resistance in peripheral tissues with insulin resistance. We may, thus, speculate that the HFHS-induced rise in IL-6 recorded in our study contributes to compromise local insulin sensitivity, while the rise in FGF-21 is a defensive mechanism to counteract the deleterious effects of glucotoxicity. Accordingly, the reduction in IL-6 levels by pHBSP might be a consequence of the EpoR/ β cR complex activation, which exerts beneficial impact on insulin sensitivity. In contrast, FGF-21 levels were not directly modulated by pHBSP. Thus, the FGF-21 reduction afforded by pHBSP may well be secondary to a reduced glucotoxicity following pHBSP, but this hypothesis warrants further investigation. It is noteworthy that our results do not argue against the role of EPO as potential regulator of adipose tissue inflammation in diet-induced obesity and insulin resistance, as recently suggested by Alnaeeli *et al* (Alnaeeli *et al.*, 2014). Instead, our report implicates that modulation of local inflammation in

skeletal muscle may be an additional mechanism by which the EPO derivative affect the actions of insulin in skeletal muscle.

Finally, we evaluated the effects of pHBSP on skeletal muscle mitochondrial function. Although mitochondrial dysfunction has been indicated as a cardinal feature of insulin resistance, most recent evidence suggest that an high caloric intake may develop insulin resistance, even in the presence of increased mitochondrial production in muscle (Garcia-Roves *et al.*, 2007; Hancock *et al.*, 2008). Our results show that HFHS feeding resulted in increases in skeletal muscle expression of COI and PGC-1 α , although no effects were observed on the expression of NRF1 (a transcription factor involved in controlling the expression of nuclear genes encoding for mitochondrial proteins) and mtTFA (a transcription factor involved in controlling the expression of mitochondrial genes) (Hock *et al.*, 2009; Scarpulla, 2008). This slight increase in selective markers of mitochondrial number and/or function detected in the skeletal muscle of HFHS mice may be suggestive of an early and weak compensatory mechanism to the deleterious consequences of dietary manipulation, which was dramatically improved in response to the activation of the EpoR/ β cR complex, as shown by a further induction in PGC-1 α expression as well as a robust up-regulation of NRF1 and mtTFA in the presence of chronic pHBSP administration.

In conclusion, chronic treatment with pHBSP, an EPO-derivative devoid of any hematopoietic effects, triggers an amelioration of the metabolic abnormalities evoked by exposure to a diet containing high concentrations of both fat and sugar. The present study also increases our understanding of the mechanism of action of pHBSP and highlights a pivotal role for the muscle EpoR/ β cR complex as a key interface between impaired insulin pathway and local inflammatory response. The demonstration that an EPO derivative that is devoid of any erythropoietin activity (that are, hence, potentially translatable to man) may act on diet-induced metabolic derangements points to a new pathway for the development of novel drugs for patients with diabetes and obesity, two closely linked healthcare challenges of modern societies that continue to rise in prevalence, with important health and economic implications.

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Author Contributions

M.C. designed the study, performed the experiments, analyzed and researched data, and wrote the manuscript. E.B., M.R., F.C. and D.N. researched data. J.C.C. performed the kidney and liver histopathological examination. R.M. analyzed and researched data, contributed to the study design, to analysis and interpretation of data. M.A., R.F. and M.A.M. contributed to discussion, and reviewed the manuscript. C.T. designed the study, contributed to drafting and revising the manuscript and edited the final version. M.C. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All the authors approved the final version.

Conflicts of interest

None

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TABLE 1

Effects of diet and pHBSP chronic administration on metabolic parameters

	Control	Control+pHBSP	HFHS	HFHS+pHBSP
	<i>(n = 12)</i>	<i>(n = 6)</i>	<i>(n = 12)</i>	<i>(n = 12)</i>
Body weight, g	29.05±0.58 [§]	28.7±1.21 [§]	38.00±0.82*	34.7±0.73* [§]
Body weight gain, g	9.17±0.75 [§]	8.14±1.67 [§]	16.21±1.51*	10.03±1.90 [§]
Food intake, g/die	3.80±0.40	3.20±0.50	2.90±0.40	2.70±0.40
Caloric intake (kcal/die)	10.80±0.92 [§]	9.92±1.55 [§]	14.05±0.80*	12.25±0.80
Epididymal fat weight (%BW)	2.41±0.09	2.11±0.21	3.34±0.25*	2.52±0.25 [§]
Kidney weight (%BW)	1.06±0.05	1.08±0.09	1.19±0.08*	1.08±0.05 [§]
Liver weight (%BW)	4.93±0.25	4.59±0.45	5.23±0.28	4.88±0.31
Gastrocnemius weight (%BW)	1.04 ±0.08	1.08 ± 0.11	1.22 ± 0.06	1.09 ± 0.06

Data are means±S.E.M.

* p< 0.01 vs Control

§ p< 0.05 vs HFHS

BW: body weight

TABLE 2

Effects of diet and pHBSP chronic administration on mouse blood chemistry

	Control	Control+pHBSP	HFHS	HFHS+pHBSP
	(<i>n</i> = 12)	(<i>n</i> = 6)	(<i>n</i> = 12)	(<i>n</i> = 12)
Triglycerides, <i>mmol/L</i>	0.52±0.08 [§]	0.54±0.09 [§]	1.02±0.07*	0.63±0.03 [§]
Total cholesterol, <i>mmol/L</i>	2.30±0.09 [§]	2.54±0.11	2.66±0.09*	2.16±0.08 [§]
LDL, <i>mmol/L</i>	0.65±0.17 [§]	0.61±0.09 [§]	1.08±0.18*	0.78±0.09 [§]
HDL, <i>mmol/L</i>	1.83±0.14	1.81±0.25	1.19±0.17*	2.04±0.17 [§]
Adiponectin, <i>ng/mL</i>	12.32±0.69 [§]	11.92±1.18 [§]	7.51±0.35*	10.76±0.63 [§]
Leptin, <i>pg/mL</i>	33.56±2.06 [§]	34.53±2.87 [§]	58.36±3.87*	38.78±6.81 [§]

Data are means±S.E.M.

* *p* < 0.01 vs Control[§] *p* < 0.01 vs HFHS

FIGURE LEGENDS:

Figure 1. Effects of high-fat high-sugar (HFHS) diet and chronic administration of pHBSP (30 $\mu\text{g/kg}$, *s.c.*) on serum insulin (Panel A), blood glucose (Panel B) and oral glucose tolerance (Panel C). Values are mean \pm S.E.M of 6 animals per group. ★ $P < 0.05$ vs Control.

Figure 2. Effects of high-fat high-sugar (HFHS) diet and pHBSP administration on kidney structure and function. Representative pictures (Panel A) from mice maintained on the control diet or the HFHS diet and treated with pHBSP (30 $\mu\text{g/kg}$, *s.c.*). Kidneys from mice fed with a standard laboratory chow showed a normal appearance in the presence or absence of drug treatment. The HFHS diet produced an intense vacuolar degeneration of the S1 and S2 portions of the proximal convoluted tubules. These alterations were significantly attenuated by pHBSP administration. (arrows point S1 and S2 portions of the proximal convoluted tubules; asterisks indicate tubular cells with vacuolar degeneration). Urinary albumin:creatinine ratio (ACR; Panel B) and blood urine nitrogen (BUN; Panel C) levels were measured in mice exposed to the control diet or the HFHS diet in the absence or presence of pHBSP (30 $\mu\text{g/kg}$, *s.c.*). Values are mean \pm S.E.M of 6 animals per group. ★ $P < 0.05$ vs Control ● $p < 0.05$ versus HFHS.

Figure 3. Effects of high-fat high-sugar (HFHS) diet and pHBSP administration on liver lipid accumulation. Panel A: representative $\times 40$ magnification photomicrographs of Oil Red O staining on liver sections from mice maintained on the control diet or the HFHS diet and treated with pHBSP (30 $\mu\text{g/kg}$, *s.c.*). Arrows point hepatocytes with lipid droplets. Panel B: triglyceride (TG) content in mouse liver. Data are mean \pm S.E.M of 6 animals per group. ★ $P < 0.05$ vs Control ● $p < 0.05$ versus HFHS.

Figure 4. Alterations in insulin signalling pathway in the skeletal muscle of mice maintained on HFHS diet in the absence or presence of pHBSP. Total protein expression of IRS-1 (Panel A), Akt (Panel B), and GSK-3 β (Panel C) as well as their related phosphorylated forms were analyzed by Western blot on the gastrocnemius homogenates obtained from mice exposed to the control diet or the HFHS diet and treated with pHBSP (30 μ g/kg, *s.c.*). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized to the Control band. The data are means \pm S.E.M. of three separate experiments. ★ $p < 0.05$ versus Control; ● $p < 0.05$ versus HFHS.

Figure 5. Effects of dietary manipulation and pHBSP treatment on GLUT-4 localization (Panel A, original magnification: 400x) and expression (Panel B), AS160 total protein expression and Thr⁶⁴² phosphorylation (Panel C) and glycogen content (Panel D) in the skeletal muscle from mice exposed to the control diet or the HFHS diet and treated with pHBSP (30 μ g/kg, *s.c.*). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin and normalized using the related Control band. Data are means \pm S.E.M. of three separate experiments for Western blot analysis and 6-8 animals per group for skeletal muscle glycogen content. ★ $p < 0.05$ versus Control; ● $p < 0.05$ versus HFHS.

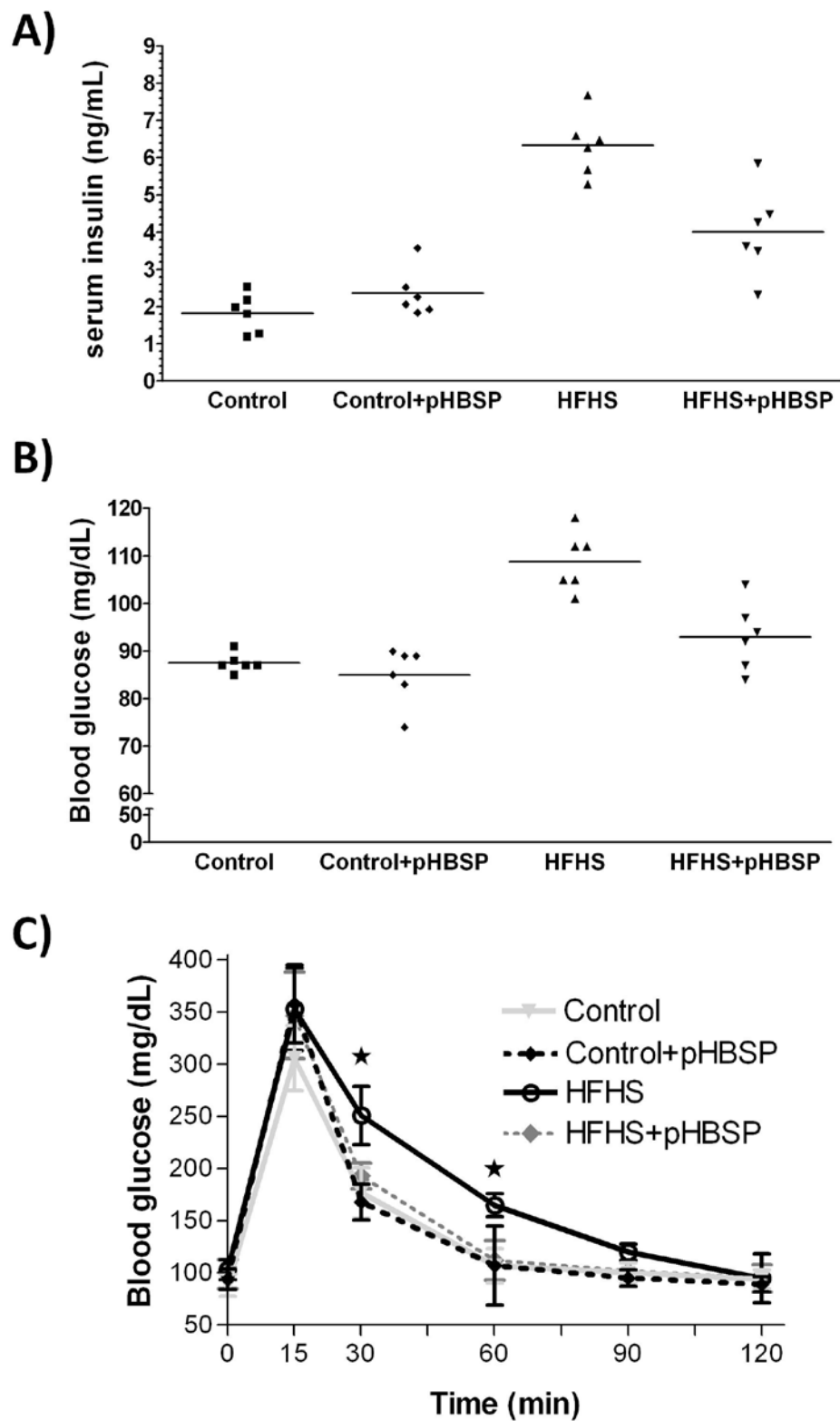
Figure 6. Effects of pHBSP on local and systemic levels of IL-6 and FGF-21 in mice fed with a HFHS diet. IL-6 and FGF-21 concentrations were analyzed by ELISA in gastrocnemius homogenates (Panels A and B, respectively) and serum (Panels C and D, respectively) of mice maintained on the control or the HFHS diet and treated with pHBSP (30 μ g/kg, *s.c.*). Data are means \pm S.E.M. of 6-8 animals/group. ★ $p < 0.05$ versus Control; ● $p < 0.05$ versus HFHS.

Figure 7. Effects of pHBSP treatment on markers of mitochondrial production/biogenesis in the gastrocnemius of mice fed with a HFHS diet. Cytochrome c oxidase-1 (COI, Panel A), peroxisome

proliferator-activated receptor γ co-activator 1- α (PGC1- α , Panel B), mitochondrial transcription factor A (mtTFA, Panel C) and nuclear respiratory factor-1 (NRF-1, Panel D) protein expression was analysed by Western blot on the gastrocnemius homogenates obtained from mice maintained on the control or the HFHS diet and treated with pHBSP (30 μ g/kg, *s.c.*). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized using the related Control band. The data are means \pm S.E.M. of three separate experiments. ★ $p < 0.05$ versus Control; ● $p < 0.05$ versus HFHS.

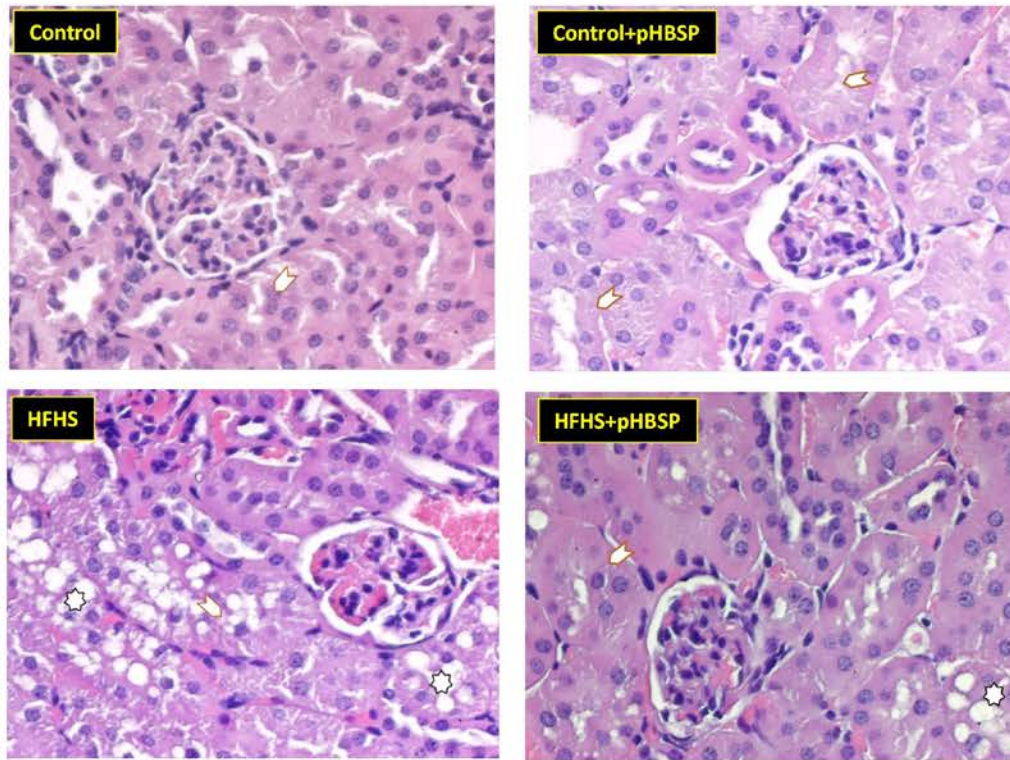
Figure 8. Heterodimerization between EpoR and β cR in the mouse skeletal muscle. Co-immunoprecipitation analysis illustrating the heteromeric complex consisting of EpoR and β cR in gastrocnemius homogenates obtained from mice maintained on the control or the HFHS diet and treated with pHBSP (30 μ g/kg, *s.c.*). Lysate from skeletal muscles were subjected to immunoprecipitation (IP) with anti-EpoR antibody and then exposed to immunoblot (IB) analysis with either anti-EpoR (left panel) or anti- β cR antibodies (right panel). The results, representative of two independent experiments, show interaction between the two receptors both in control condition and after dietary manipulation and/or pHBSP treatment.

Figure 1

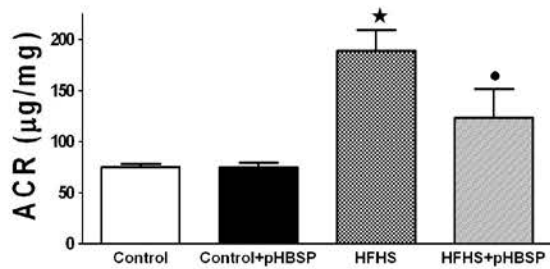


A)

Figure 2



B)



C)

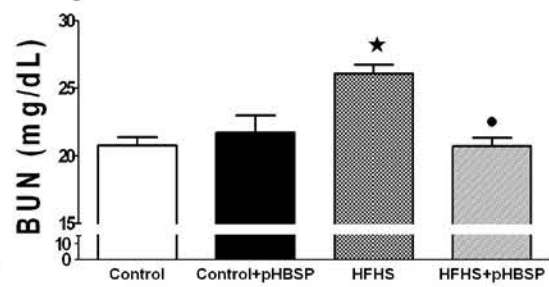
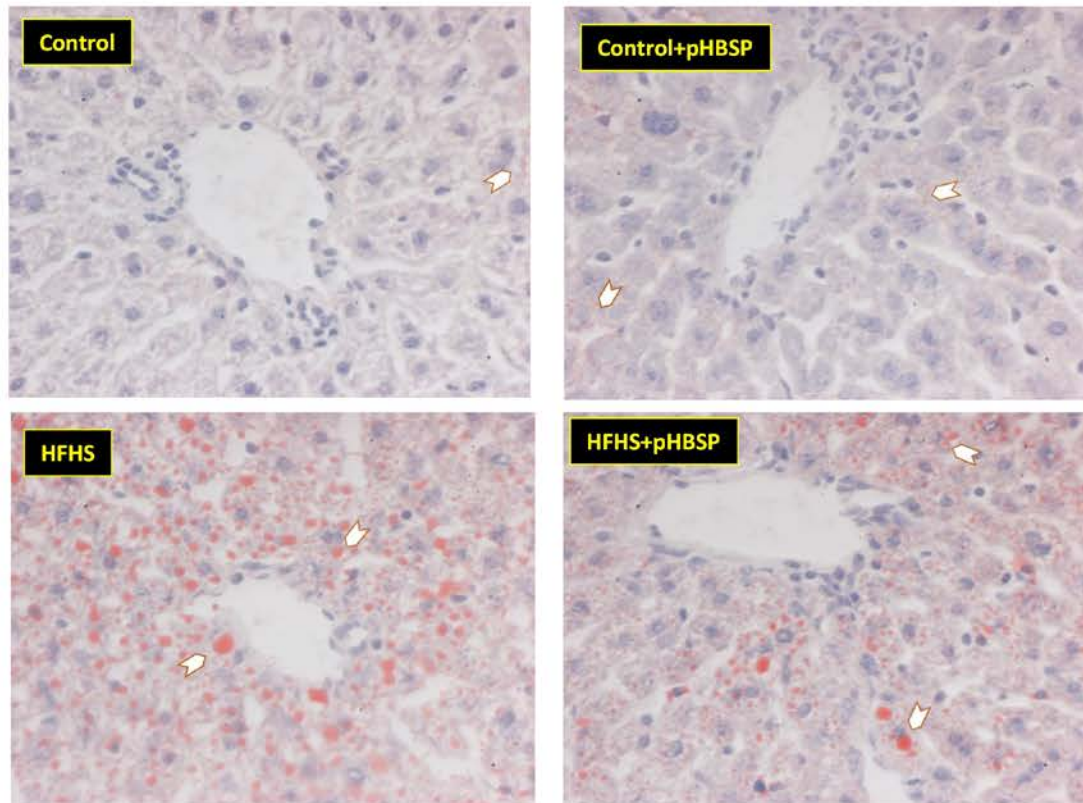


Figure 3

A)



B)

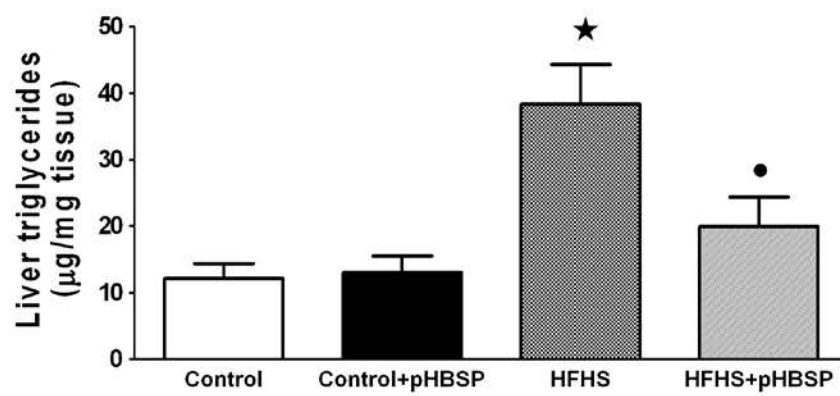
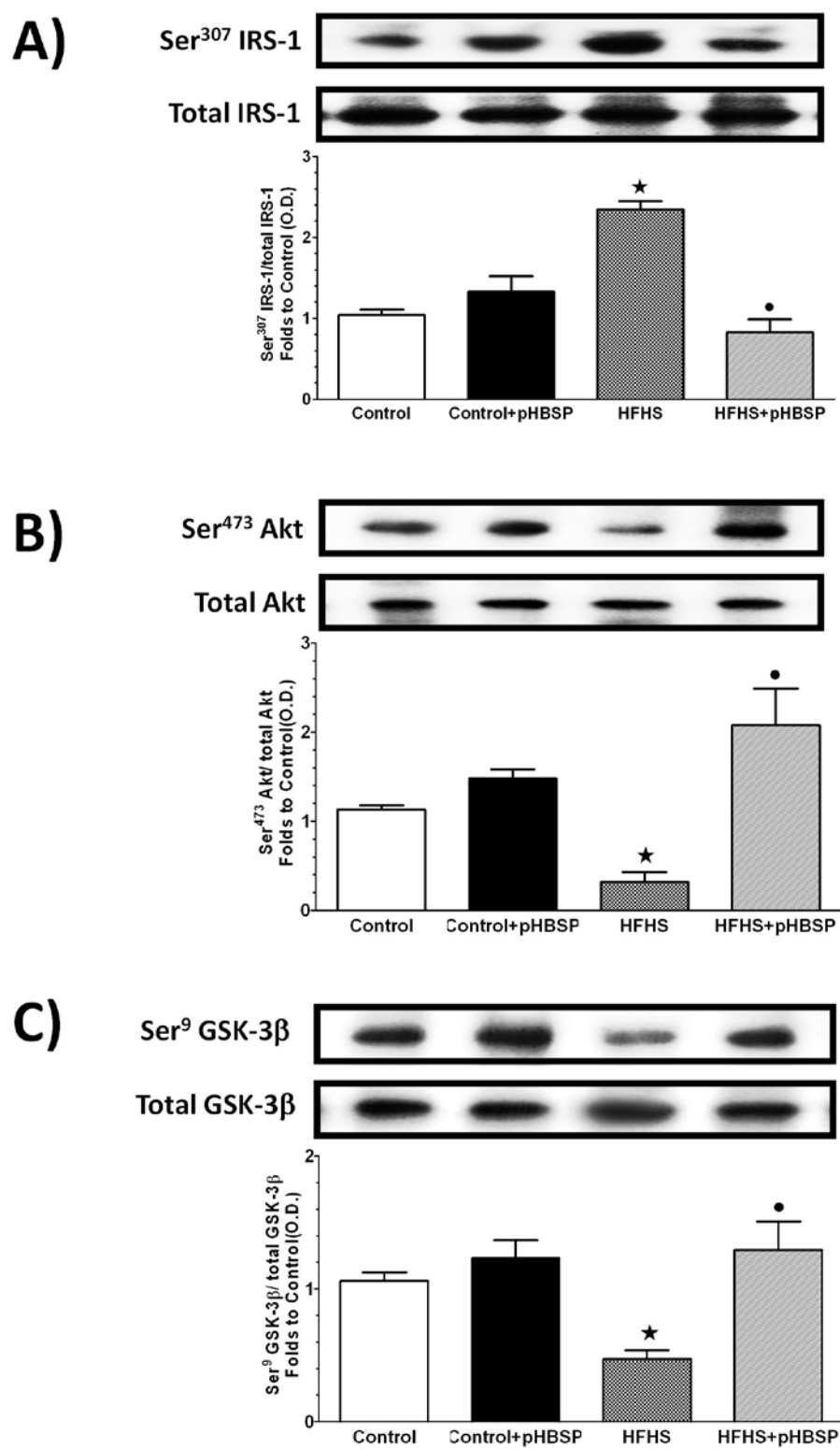
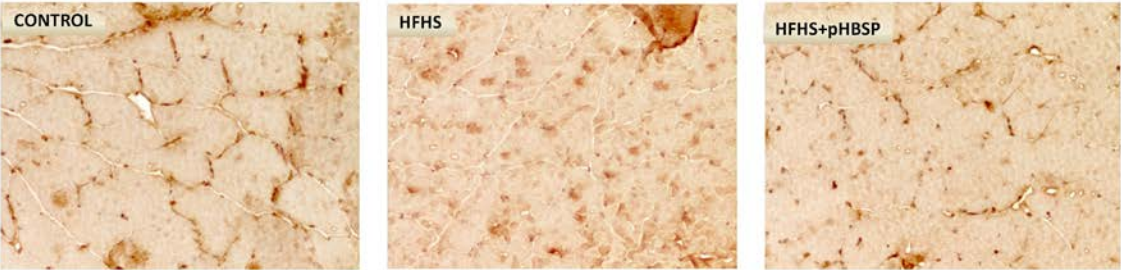


Figure 4

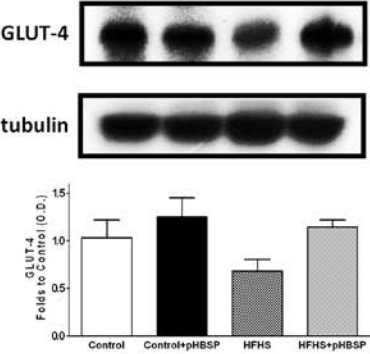


A)

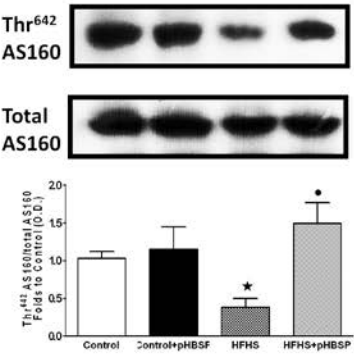
Figure 5



B)



C)



D)

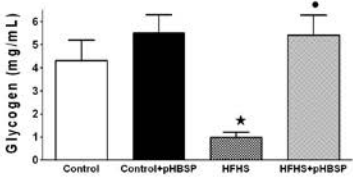


Figure 6

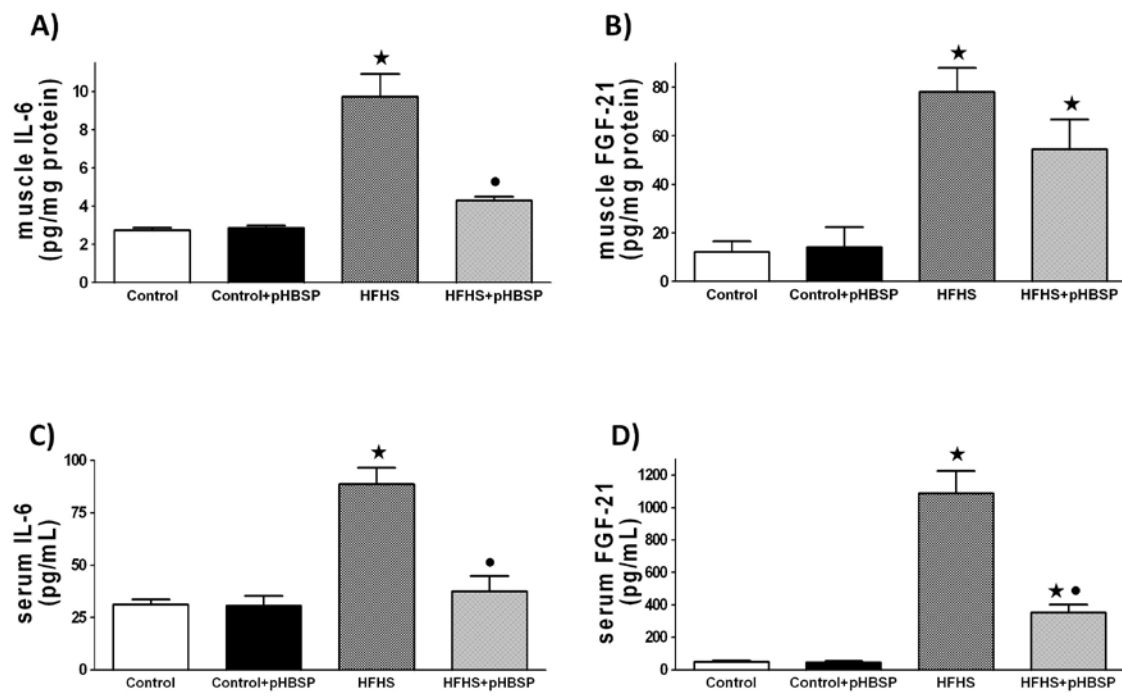


Figure 7

